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Primary Structure and Functional Expression of the Human Receptor for *Escherichia coli* Heat-stable Enterotoxin*

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Heat-stable enterotoxin (STa) produced by *Escherichia coli* induces intestinal secretion in mammals by binding to the brush border membrane of the small intestine and activating guanylyl cyclase. We report here the cloning and expression of a cDNA encoding the human receptor for STa. The receptor contains both an extracellular ligand binding site and a cytoplasmic guanylyl cyclase catalytic domain, making it a member of the same receptor family as the natriuretic peptide receptors. Stable mammalian cell lines overexpressing the STa receptor specifically bind ^{125}I-STa (K_d ~1.0 nM) and respond to STa by dramatically increasing (~50-fold) cellular cGMP levels. Sequence comparisons between the human and the rat STa receptors show less conservation in the extracellular domain than similar comparisons of natriuretic peptide receptors. This divergence may indicate important species differences in ligand-receptor interaction.

Escherichia coli heat-stable enterotoxins (STa)[1] are a family of homologous peptides that can cause various diarrheal diseases, including traveler's diarrhea and epidemic diarrhea in newborns (1, 2), accounting for as much as 50% of infant mortality in underdeveloped countries (3). STa has been shown to bind specific high affinity receptors located on the brush border membrane of the small intestine (4, 5). Interaction of STa with its receptor is reported to stimulate guanylyl cyclase activity (6–8). The subsequent increase in intracellular cyclic GMP (cGMP) induces fluid secretion primarily by impairing sodium and chloride absorption across the brush border membrane while stimulating the secretion of chloride and water (6, 9, 10).

Although the biological processes that follow the binding of STa are poorly understood, it has been proposed that the STa receptor and the guanylyl cyclase are two different proteins that are coupled by cytoskeletal components (11, 12). However, the concept of receptor-guanylyl cyclases has emerged recently with the cloning and expression of cDNAs encoding cell surface receptors for the atrial natriuretic peptides (13–16). These studies have demonstrated that the binding sites for the hormones and the guanylyl cyclase catalytic activity reside on the same protein. The two identified natriuretic peptide receptor guanylyl cyclases (ANPRA and ANPRB) have a common architecture. A single transmembrane domain separates the extracellular or binding portion of the receptors from the cytoplasmic domains which can be divided in two parts of approximately equal size. Immediately adjacent to the transmembrane domain is the kinase homology domain that displays sequence homology with the catalytic domains of protein kinases (17). The carboxyl termini of the two natriuretic peptide receptors contain the guanylyl cyclase catalytic activity based on their homology to the soluble guanylyl cyclases (14, 15) and on detection of guanylyl cyclase activity in extracts from bacteria producing the carboxy terminal domain of ANPRA (18).

Recently, using low stringency polymerase chain reaction conditions with degenerate oligonucleotides designed from a highly conserved region within the guanylyl cyclase domains of the natriuretic peptide receptors, Schulz and collaborators (19) have identified a new membrane bound guanylyl cyclase in the rat. This intestinal-specific receptor which is not homologous to the natriuretic peptide receptors in the extracellular domain, was identified as the rat STa receptor. In this paper we report the characterization of the cDNA encoding the human receptor for the *E. coli* STa enterotoxin (STaR). Cells overexpressing STaR bind STa with high affinity, show marked elevation of intracellular cGMP content in response to STa and provide an important model system to study the molecular mechanism of action of STa.

MATERIALS AND METHODS

Isolation and Sequencing of Human STa Receptor cDNA—Total RNA was isolated from a surgical specimen of human ileum using RNAzol (Cinn/Biotex). Five μg of RNA was reverse-transcribed using 1000 units of avian myeloblastosis virus reverse transcriptase (United States Biochemical Corp.) and 5 μg of oligo(dT) as primer. Ten percent of the resulting cDNA was used as template for a polymerase chain reaction containing 10 mM Tris-Cl pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.2 mM of each deoxynucleotide, and 0.2 mM of two degenerate primers: antisense 5'GCTAAAAATAGGTGCTATCG-TGGTC(T/C)G(A/T)(C/A)TT3' and sense 5'TGTGGGAGGAGGA-TCCAGAAAAGA(A/C)G(A/G)CCC3', synthesized based on the sequence of the rat STaR (19). The product of the reaction, a 1 kilobase pair fragment corresponding to the 3' end of the coding region of the rat STaR sequence (amino acids 706–1052), was gel-purified, labeled to high specific activity by random priming, and used to screen 10^6 clones of a human terminal ileum cDNA library (provided by John McLean, Genentech, Inc.) in λgt10. Duplicate filters were hybridized under high stringency conditions at 42 °C in 50% formamide, 5 × SSC, 10 × Denhardt's, 0.05 M sodium phosphate (pH 6.5), 0.1% sodium pyrophosphate, 50 μg/ml of sonicated salmon sperm DNA, and 10% dextran sulfate. Filters were rinsed in 2 × SSC at 42 °C and then washed twice in 0.2 × SSC, 0.1% sodium dodecyl sulfate at 42 °C. Hybridizing phage were plaque-purified, and the largest cDNA inserts were subcloned into the Bluescript plasmid (Stratagene). Both strands were sequenced by the dideoxy chain termination procedure

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank[TM]/EMBL Data Bank with accession number(s) M73489.

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[1] The abbreviations used are: STa, heat-stable enterotoxin; ANPR, atrial natriuretic peptide receptor; EBV, Epstein-Barr virus; DMEM, Dulbecco's modified Eagle's medium; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

(20). Sequence homology alignments were performed by the method of Hein (21).

STa Receptor Expression—A full-length cDNA fragment, from the 5′ *Xho*I cloning linker to *Hind*III (position 3344, Fig. 1), was subcloned into the plasmid pNebo, an Epstein-Barr virus (EBV)-based vector (22) containing a resistance marker for neomycin, the EBV origin of replication, and the EBV nuclear antigen gene EBNA-1.[2] The cDNA insert is under the control of the cytomegalovirus immediate early promoter.

Human embryonic kidney 293 cells maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with F-12 nutrient mixture, 20 mM Hepes (pH 7.4), and 10% fetal bovine serum were transfected by the calcium phosphate method as described (23). Selection for neomycin-resistant cells was begun 48 h after transfection with Geneticin (GIBCO) at 400 μg/ml. Ten days later, resistant colonies were pooled and kept under selection until analysis.

cGMP Assay—Cells expressing the STaR were plated in 6-well dishes (4×10^5 cells/well). Forty-eight hours later, cells were washed once with DMEM containing 20 mM Hepes (pH 7.2), preincubated with DMEM containing 20 mM Hepes, 0.1 mM isobutylmethylxanthine for 10 min at 37 °C, and incubated for 10 min at 37 °C in DMEM containing 20 mM Hepes, 0.1 mM isobutylmethylxanthine, and various concentrations of *E. coli* STa (Sigma). The incubation media was aspirated and replaced with 1 ml of 10% trichloroacetic acid, and the cells were frozen quickly on dry ice. After the samples thawed at room temperature, the cell debris was removed by centrifugation at 3500 × *g* for 10 min. Samples were extracted three times with 0.5 ml of water-saturated ether and warmed to 55 °C for 20 min to evaporate the residual ether. Aliquots were acetylated and analyzed for cGMP concentration by radioimmunoassay according to the manufacturer's instructions (Biomedical Technologies Inc., Stoughton, MA).

^{125}I-STa Binding Assay—STa was radioiodinated to a specific activity of ~2000 Ci/mmol using $Na^{125}I$ and lactoperoxidase (24). [^{125}I-Tyr]STa was purified by reverse phase high performance liquid chromatography as described (25). Transfected cells were washed 3 times with PBS, harvested with PBS + 1 mM EDTA and washed with PBS containing 0.1% bovine serum albumin, 0.02% sodium azide (PBSA buffer). Duplicate samples of 5×10^5 cells in 0.5 ml of PBSA were incubated for 2 h at room temperature while shaking with 50 pM ^{125}I-STa plus various concentrations of unlabeled STa. The assay was terminated by rapid filtration through Whatman GF/C filters previously treated with 1% polyethyleneimine. Filters were rinsed three times with 1 ml of PBS and counted in a γ spectrometer (Iso-Data 100).

RESULTS

STa Receptor cDNA Cloning—Degenerate oligonucleotides corresponding to two regions of the rat STa receptor cDNA (19) were synthesized and used to amplify human ileum cDNA by polymerase chain reaction. A 1-kilobase pair fragment representing the 3′ end of the human STaR coding sequence was isolated and used as an homologous probe to screen a human terminal ileum cDNA library of 10^6 clones. Thirty-four positive phage plaques were obtained and the clone (No. 10) with the largest insert was used for nucleotide sequence determination (Fig. 1). The cDNA covers 3745 base pairs and encompasses the entire coding region for human STaR.

An ATG codon within a favorable consensus sequence for eukaryotic translation initiation (26) is present at nucleotide 49–51 and defines the beginning of an open reading frame of 1073 amino acids. The hydrophobic amino-terminal residues probably constitute a signal peptide which, according to the consensus rules of von Heijne (27), is probably cleaved after residue 23. This would generate a mature protein of 1050 residues with a predicted M_r of 120,707. The open reading frame is terminated by a TAA stop codon followed by 475 base pairs of 3′-untranslated sequence. A consensus polyadenylation signal AAUAAA (28) is present at position 3731. Hydrophobicity analysis predicts a single transmembrane domain from residue 408 to 431 that is followed by the stop transfer sequence Arg-Lys (29). The 407-amino acid extracellular domain contains nine potential *N*-linked glycosylation sites and 8 cysteines. The cytoplasmic domain is 620 amino acids long and contains 10 cysteine residues.

Sequence Comparisons—The overall amino acid sequence identity between the human STaR and the rat STaR is 81%, but this homology is not evenly distributed (Fig. 2). The 620 amino acid cytoplasmic regions are 91% identical, whereas the extracellular domains are 71% identical. Seven out of nine potential *N*-linked glycosylation sites are conserved and all 8 cysteine residues of the human extracellular domain are also present in the rat sequence. The other members of the guanylyl cyclase receptor family, the human atrial natriuretic peptide receptors A (ANPRA, Ref. 15) and B (ANPRB, Ref. 13) (Fig. 3) have three distinct domains that are defined on the basis of amino acid sequence homology. These are the extracellular ligand binding domain and the intracellular kinase homology domain and guanylyl cyclase catalytic domain. The extracellular domain of the human STaR is only 17 and 15% homologous to ANPRA and ANPRB, respectively, whereas ANPRA and ANPRB are 43% identical. Two potential *N*-glycosylation sites and 4 cysteines are conserved between STaR and ANPRA. One potential *N*-glycosylation site and 3 cysteines are conserved between STaR and ANPRB.

In the intracellular region, the kinase homology domain of the STaR is 37 and 36% identical to the equivalent domains of ANPRA and ANPRB, respectively. Notably, as seen with the rat STaR, the consensus GXGXXG nucleotide binding motif present in protein kinases and ANPRA (17) is not conserved. The guanylyl cyclase catalytic domain has 53% identity with both ANPRA and ANPRB catalytic domains.

Expression and Characterization of STaR—To confirm that our cDNA clone encoded the human STa receptor, a cDNA fragment containing the entire open reading frame was inserted under the transcriptional control of the cytomegalovirus immediate-early promoter in the EBV-based plasmid pNebo. The pNebo-STaR expression vector was stably transfected into the human embryonic kidney 293 cell line. Neomycin-resistant transfectants were incubated in the presence of increasing concentrations of *E. coli* STa for 10 min and the intracellular content of cGMP determined (Fig. 4). In unstimulated cells, 0.25 pmol of cGMP/10^6 cells was detected. When stimulated with 5 μM of STa, the cGMP content increased 48-fold to 12 pmol/10^6 cells. Half-maximal accumulation (EC_{50}) of intracellular cGMP is seen at a concentration of 150 nM STa. No elevation of the intracellular content of cGMP levels was observed when these STaR-expressing cells were stimulated with the natriuretic peptides ANP, brain natriuretic peptide, or C-type natriuretic peptide. In addition, STa stimulates only cells expressing the STaR and not cells expressing ANPRA or ANPRB (data not shown).

Specific binding of ^{125}I-STa to intact cells transfected with pNebo-STaR or with pNebo was measured in presence of increasing concentrations of unlabeled STa (Fig. 5). Specific competitive binding was observed for the STaR-expressing cells, whereas no binding over background was detected in pNebo-transfected cells. Scatchard analysis of the displacement data demonstrated the presence of a single high affinity binding site with a calculated K_d of approximately 1 nM (Fig. 5, *inset*). This value is comparable with the 1.4 nM K_d determined for ^{125}I-STa binding to rat brush border membranes (11).

DISCUSSION

The human STaR reported in this study has the same overall structural organization as the natriuretic peptide

[2] G. Cachianes and D. Leung, unpublished data.

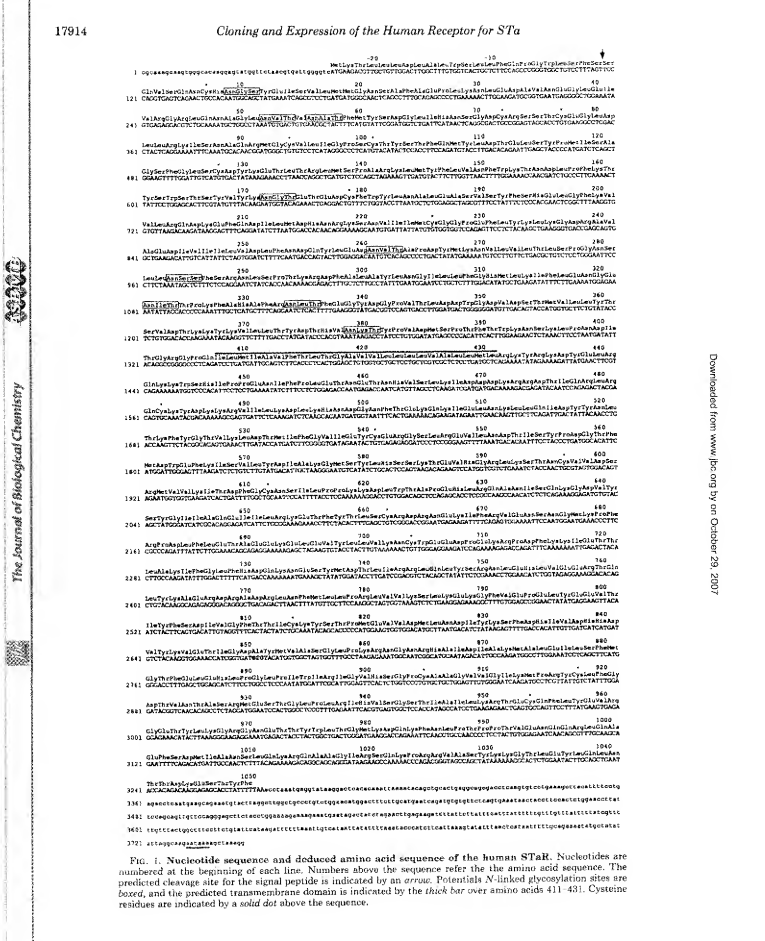

FIG. 1. **Nucleotide sequence and deduced amino acid sequence of the human STaR.** Nucleotides are numbered at the beginning of each line. Numbers above the sequence refer the the amino acid sequence. The predicted cleavage site for the signal peptide is indicated by an *arrow*. Potential *N*-linked glycosylation sites are *boxed*, and the predicted transmembrane domain is indicated by the *thick bar* over amino acids 411–431. Cysteine residues are indicated by a *solid dot* above the sequence.

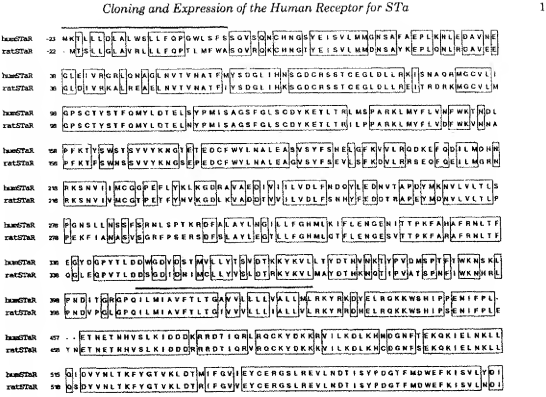

FIG. 2. **Amino acid sequence homology between the human and the rat STaR.** The predicted amino acid sequences of the human STaR is aligned with rat STaR (18). Gaps introduced for optimal alignment are shown by *dashes*. Identical amino acids are *boxed*. The signal sequence and the transmembrane domain are shown by *overlines*.

receptors ANPRA and ANPRB, adding a new member to the guanylyl cyclase receptor family. These receptors contain a single transmembrane domain that divides the molecule into an extracellular hormone binding region and an intracellular signalling domain. The intracellular domain contains a region of about 250 amino acids that has strong homology to the catalytic domain of many protein kinases. In the case of ANPRA, this domain has been shown to be required for ligand-dependent signaling (30). It is unclear, however, if this domain has intrinsic kinase activity.

Based on alignment of the amino acid sequences of STaR, ANPRA and ANPRB, the most conserved region is the guanylyl cyclase domain (~50% identity), followed by the kinase homology domain (~35% identity). The extracellular domain is less conserved with only 15 and 17% similarity with ANPRA and ANPRB, respectively. This low homology between the different extracellular domains suggests that the natural ligand for the STaR will probably not be a member of the natriuretic peptide hormons family. Moreover, none of the natriuretic peptides identified as yet (ANP, BNP, and CNP) is able to stimulate guanylyl cyclase activity in human 293 cells overexpressing the STaR. STa, on the other hand, stim-

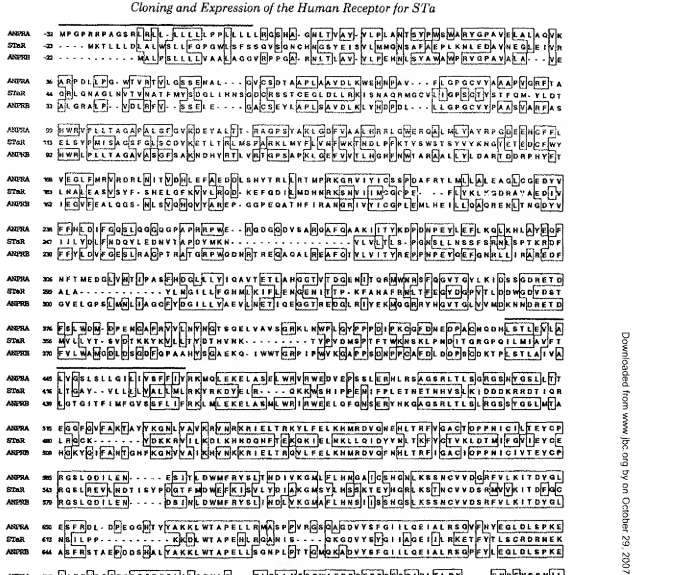

FIG. 3. **Sequence homology between the guanylyl cyclase receptors.** Deduced amino acid sequence of the human STaR is aligned with the human ANPRA (15) and the human ANPRB (13) amino-acid sequences. Gaps introduced for optimal alignment are shown by *dashes*. Identical amino acids are *boxed*. The signal sequence and the transmembrane domains are shown by an *overline*.

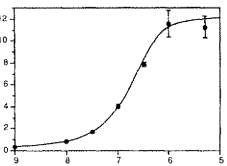

FIG. 4. **Dose-dependent stimulation of STaR overexpressing cells by STa.** Human 293 cells stably transfected with the pNebo-STaR expression plasmid were incubated with various concentrations of STa for 10 min. Intracellular cGMP accumulation was then determined. Each point represents the mean of triplicate samples assayed in duplicate and are represented ± S.E.

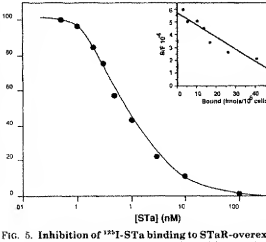

FIG. 5. **Inhibition of ^{125}I-STa binding to STaR-overexpressing cells.** Varying concentrations of unlabeled STa were incubated with 50 pM ^{125}I-STa and 5×10^5 G418-resistant 293 cells expressing STaR. Nonspecific binding was determined in the presence of 500 nM of STa. The percent specific binding is plotted *versus* the concentration of STa. Each point represents the mean of duplicate determination. A Scatchard analysis of the data is presented in the *inset*.

ulates cGMP accumulation in these cells with an EC_{50} of approximately 150 nM and binds with an affinity of ~1 nM.

The cDNA that we describe here encodes a receptor with both the STa binding site and the guanylyl cyclase catalytic site. These data are not consistent with previous results, indicating that the binding site and the catalytic site could be dissociated (11, 12). A large number of proteins have been identified in cross-linking experiments with STa (31), and our results do not rule out the possibility of heterogeneity among STa receptors. However, cross-linking experiments on opossum kidney cells with ^{125}I-STa reveal a single protein with an M_r of 120,000 (35), the predicted size for the STaR we report here.

Comparison of the predicted amino acid sequences of the human and the rat STaR sequences shows that the intracellular domain is more highly conserved (91% identity) than the extracellular domain (71% identity). The entire predicted amino acid sequences of ANPRA (92%) and ANPRB (98%) are highly conserved between rat and human, including all the glycosylation sites. This divergence of the STaR extracellular domain might be expected to reflect some species-specific interactions with the ligand but there appears to be little difference between the human receptor reported here and the rat receptor (12) interaction with a 19-amino acid STa peptide from *E. coli*. However, a putative endogenous ligand for these receptors may be species-specific.

The stable cell line expressing human STaR cDNA provides a useful tool to identify such a ligand or to design possible antagonists of the undesirable actions of STa. Antagonists that target the human receptor might be of therapeutic interest. To begin designing possible antagonists, information about the active portion of the STa peptide is valuable. In fact, it has been shown that only 13 amino acids out of the 19 or 20 are shown to be necessary for activity in suckling mice (32). This 13-amino acid sequence is very well conserved between the various bacteria that secrete heat-stable enterotoxins and is characterized by the presence of 6 cysteines, all of which are essential for activity (33). A large number of small peptides, known as conotoxins, have been purified from sea snail venoms (34) and found to have sequence similarity with the active portion of the heat-stable enterotoxins. However, one of the most homologous conotoxin, conotoxin GI (46% identity with the active portion of STa), does not bind to the STaR present on the rat intestinal epithelium (12).

STa stimulation of guanylyl cyclase was observed recently in various opossum epithelial cells other than intestine (35). This result suggests that an endogenous ligand for the STa receptor regulates guanylyl cyclase activity in cells that would not normally be exposed to STa. However, non-intestinal distribution of STaR has not been reported for other mammals. In the case of the rat, STaR mRNA was detected by northern blot analysis only in the intestine (19). Detailed information on the tissue-specific expression of this receptor await additional techniques such as *in situ* hybridization. Availability of cells lines overexpressing the STaR may help to identify the existence of a putative natural agonist for this receptor and to elucidate the role of this guanylyl cyclase receptor in intestinal cells.

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